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Use of the response of photosynthesis to oxygen to estimate mesophyll conductance to carbon dioxide in water-stressed soybean leaves

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ABSTRACT

Methods of estimating the mesophyll conductance (g_m) to the movement of CO_2 from the substomatal airspace to the site of fixation are expensive or rely upon numerous assumptions. It is proposed that, for C_3 species, measurement of the response of photosynthesis to $[O_2]$ at limiting $[CO_2]$, combined with a standard biochemical model of photosynthesis, can provide an estimate of g_m. This method was used to determine whether g_m changed with $[CO_2]$ and with water stress in soybean leaves. The value of g_m estimated using the O_2 response method agreed with values obtained using other methods. The g_m was unchanged over the tested range of substomatal $[CO_2]$. Water stress, which decreased stomatal conductance (g_s) by about 80%, did not affect g_m, while the model parameter V_{Cmax} was reduced by about 25%. Leaves with g_s reduced by about 90% had g_m values reduced by about 50%, while V_{Cmax} was reduced by about 64%. It is concluded that g_m in C_3 species can be conveniently estimated using the response of photosynthesis to $[O_2]$ at limiting $[CO_2]$, and that g_m in soybean was much less sensitive to water stress than g_s, and was somewhat less sensitive to water stress than V_{Cmax}.

Key-words: drought; internal conductance; oxygen inhibition.

INTRODUCTION

With recent evidence that the conductance of the pathway for CO_2 movement from the intercellular airspace to the site of fixation inside the chloroplast during photosynthetic CO_2 fixation, termed mesophyll conductance (g_m), is functionally significant and is not simply a physical diffusive conductance (reviewed in Flexas *et al.* 2008; Warren 2008b), there has been renewed interest in how it may limit photosynthesis in different species (Warren & Adams 2006), with various stresses (Centritto, Loreto & Chartzoulakis 2003; Galmes, Medrano & Flexas 2007), and affect the acclimation of photosynthesis to environment (e.g. Singsass, Ort & DeLucia 2003; Ethier *et al.* 2006; Yamori *et al.* 2006; Bunce 2008). Unfortunately, methods of estimating g_m are expensive and not readily available to most researchers and/or rely upon assumptions that are difficult to prove. Among the many methods of estimating g_m (reviewed in Warren 2006), there are three basic types commonly used: discrimination among isotopes of carbon during photosynthesis, combined fluorescence and leaf gas exchange measurements, and estimates based on the curvature of the slope of the response of photosynthesis to substomatal CO_2 concentration (C_i). The instrumentation required for online measurements of carbon isotope discrimination is expensive and not available to most researchers, and estimation of g_m with this method relies upon assumptions about discrimination by non-photosynthetic processes (Evans *et al.* 1986). The method using the curvature of A versus C_i curves (Ethier & Livingston 2004) may not be appropriate if g_m varies with the $[CO_2]$, as found by Flexas *et al.* (2007) and During (2003). The two types of fluorescence estimates, the 'constant J' method (Bongi & Loreto 1989) and the 'variable J' method (Di Marco *et al.* 1990) each have limitations (discussed in Harley *et al.* 1992), sometimes disagree significantly (Bunce 2008) and may depend on which leaf surface the fluorescence signal is viewed from (Lichtenthaler, Buschmann & Knapp 2005; Bunce 2008). The interpretation of fluorescence signals in drought-stressed plants also remains uncertain (Osmond, Kramer & Luttge 1999). It is proposed that, for C_3 species, the measurement of the response of photosynthesis to $[O_2]$, for example from 2 to 21% O_2, at limiting $[CO_2]$, combined with a standard Farquhar-type biochemical model of C_3 photosynthesis (Farquhar, von Caemmerer & Berry 1980), can provide an estimate g_m that avoids many of these issues. As an example, this method was used to determine whether g_m changed with $[CO_2]$ and with water stress in soybean leaves.

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THEORY OF THE METHOD

The method relies on the fact that O_2 and CO_2 compete for RuBp at ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco), and this competition determines the rate of net photosynthesis as long as neither substrate is saturating. The sensitivity of CO_2-limited photosynthesis to a change in

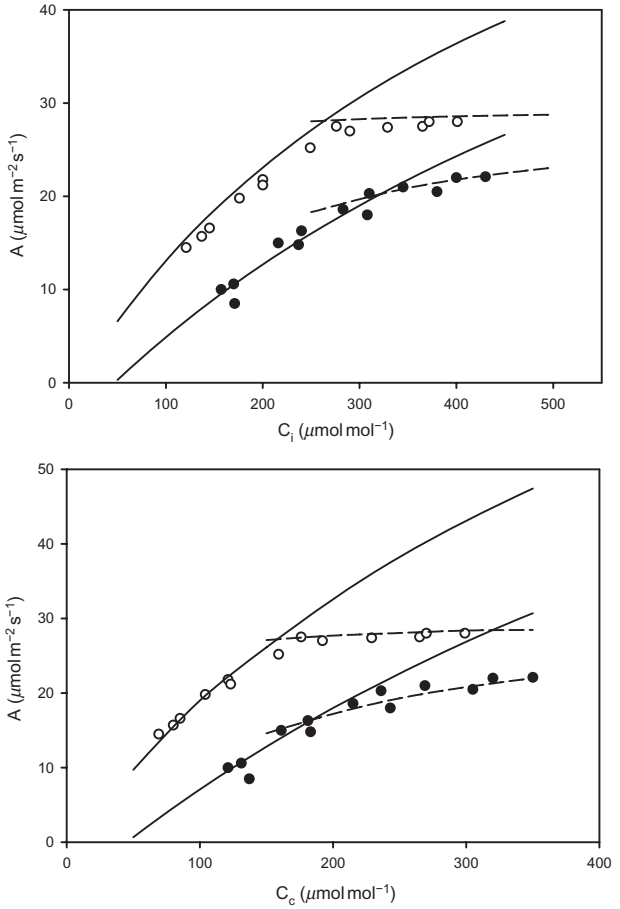

Figure 1. Net CO_2 assimilation rate (A) in relation to substomatal $[CO_2]$ (C_i) or the calculated $[CO_2]$ at ribulose 1·5-bisphosphate carboxylase/oxygenase (C_c) for three Fiskeby soybean leaves measured in 2% $[O_2]$ (open symbols) or in 21% $[O_2]$ (filled symbols). Furthermore, the predicted values of A when limited by V_{Cmax} (solid lines) or by J (dashed lines) are shown based on parameterization of the photosynthesis model with the values of A measured in 21% $[O_2]$. C_c was calculated using a constant value of g_m of 0.275 mol m^{-2} s^{-1}.

$[O_2]$ thus provides information on the $[CO_2]$ at Rubisco (C_c). As evident in the preliminary data for Fiskeby soybean, when values of V_{Cmax} and J estimated from the A versus C_i curves measured at 21% $[O_2]$ were used to calculate A at 2% $[O_2]$, the calculated rates exceeded measured rates over the whole range of C_i values (Fig. 1). When a finite value of g_m was assumed, the new values of V_{Cmax} and J estimated from the data at 21% $[O_2]$ also adequately fit the data at 2% $[O_2]$ (Fig. 1). It is not necessary to assume that the same value of g_m occurs at all C_i, because g_m can be calculated from any pair of measurements of A at two $[O_2]$, provided that A is limited by either V_{Cmax} or J at both $[O_2]$. At a given value of C_i, a finite value of g_m would lower C_c, and hence, the predicted value of A more at low than at high $[O_2]$, because of the greater $[CO_2]$ sensitivity of A at low $[O_2]$. Thus, a unique combination of higher V_{Cmax} or J and finite g_m can be found such that A at low $[O_2]$ can be predicted from A at high $[O_2]$ with a single value of V_{Cmax} or J. The procedure is illustrated in Table 1. Firstly, a value of V_{Cmax} (or J) is found, which fits the observed A at high $[O_2]$ (21% in this example) at the observed C_i. If the predicted value of A at low $[O_2]$ (2% in this example) exceeds the observed value, an arbitrary estimated value of g_m is chosen and used to calculate C_c at 21% $[O_2]$ and find the new V_{Cmax} (or J) value that fits A at 21% $[O_2]$ at that C_c. The new model value of A at 2% $[O_2]$ is then compared with the observed value at the C_c at 2% $[O_2]$. If the modelled value of A at 2% $[O_2]$ is less than the observed value, then the estimate of g_m is too low, and vice versa (Table 1).

When A versus C_i curves at both $[O_2]$ are available, one can readily pick C_i values that meet the criterion that A at both $[O_2]$ are limited by the same model parameter, V_{Cmax} or J, by comparing the observed A versus C_i curves with the photosynthesis model (Sharkey *et al.* 2007). Thus, A and C_i data at two $[O_2]$ values at different C_i values can be used to determine whether g_m changes with C_i. The main assumptions of the method are that competition at Rubisco described by the Farquhar-type C_3 photosynthesis model fully explains $[O_2]$ effects on CO_2 fixation, and that respiration in the light is unchanged over the $[O_2]$ range used. Implicit here is that g_m is not sensitive to $[O_2]$, which was tested by Loreto *et al.* (1992). A significant effect of $[O_2]$ on alternative electron sinks could potentially impact the method when used under conditions where assimilation is limited by electron transport. The importance of this to estimates of g_m has not yet been experimentally addressed.

When complete A versus C_i curves are not available, correct estimation of g_m still depends on A being limited by the same photosynthetic model parameter at both $[O_2]$. Therefore, A must be measured at two C_i at both $[O_2]$, and the parameter limiting A at each $[O_2]$ deduced by comparing observed responses of A to C_i with the photosynthesis model. Substantial errors in estimating g_m could occur if V_{Cmax} limited A at one $[O_2]$ level and J limited it at the other $[O_2]$ level, and the change in limitation was not realized in the analysis. For example, if J limited A at 21% $[O_2]$, but V_{Cmax} limited A at 2% $[O_2]$, then assuming J limitation of both rates would provide an overestimate of g_m, or no solution at all. If it were assumed that the rate at 2% $[O_2]$ was limited by V_{Cmax}, when it was actually limited by J, then g_m would be underestimated. Comparing g_m values as a

Table 1. Example of estimating g_m from the response of photosynthesis (A) to $[O_2]$ in Fiskeby soybean leaves

A measured (μmol m^{-2} s^{-1})	C_i (μmol mol^{-1})	$[O_2]$ (%)	g_m (mol m^{-2} s^{-1})	A modelled (μmol m^{-2} s^{-1})
16.0	250	21	infinite	16.0
25.3	250	2	infinite	27.1
	250	2	0.330	25.9
	250	2	0.275	25.3
	250	2	0.220	24.4

The value of g_m is that where measured and modelled values of A at 2% $[O_2]$ are equal. See text for details. Modelled values of A at 2% $[O_2]$ for g_m values ±20% of the actual value are also given.

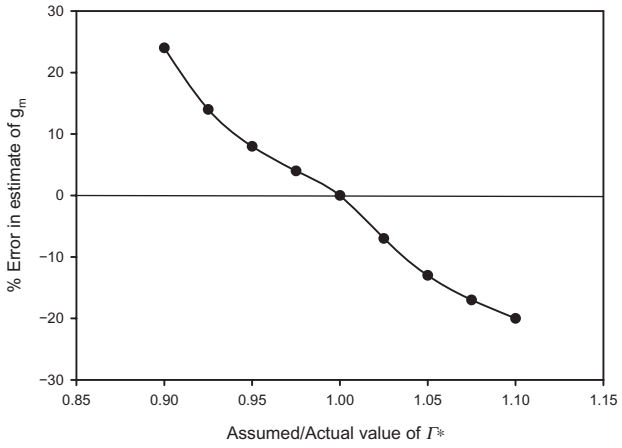

Figure 2. Errors in the estimate of g_m caused by using up to ±10% of the correct value for $\Gamma*$. This error analysis applies to the data in Fig. 1 at $C_i = 250$ μmol mol^{-1}.

function of C_i is a convenient but arbitrary strategy. If g_m varies with $[CO_2]$, it is completely unknown whether it varies with C_i, C_c or something else that co-varies with $[CO_2]$.

The precision with which g_m can be estimated for a given uncertainty in A is greater when V_{Cmax} limits A than when J limits A, since the slope of A versus C_i is much shallower when J is limiting, especially at 2% $[O_2]$ (Fig. 1). For example, g_m estimated at $C_i = 400$ μmol mol^{-1} in Fiskeby soybean would range from 0.13 to 0.65 mol m^{-2} s^{-1} for a change in A at 2% $[O_2]$ of 1 μmol m^{-2} s^{-1}. In contrast, when A is limited by V_{Cmax}, values of g_m are tightly constrained by A (Table 1).

The sensitivity of estimates of g_m to errors in K_C $(1 + O/K_O)$, Rd and $\Gamma*$ of the photosynthesis model (Sharkey *et al.* 2007) were estimated using the data for Fiskeby at a C_i of 250 μmol mol^{-1}. Errors of ±10% in K_C $(1 + O/K_O)$ produced less than a 10% error in g_m, and errors of ±10% for R_d produced less than 4% error in g_m (not shown). Errors of ±10% in $\Gamma*$ produced errors in the range of 20 to 25% in g_m (Fig. 2).

MATERIALS AND METHODS

Soybean (*Glycine max* L. Merr. cv. Fiskeby V and Essex) plants were grown one per 20 cm diameter pot in controlled environment chambers with air temperatures of 25 °C, dew point temperatures of 18 °C and 1000 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) from a mixture of high-pressure sodium and metal halide lamps for 12 h per day. Chamber $[CO_2]$ was kept between 370 and 400 μmol mol^{-1} by injecting CO_2 or CO_2-free air under the control of an infrared CO_2 analyser that sampled chamber air continuously. Pots were filled with vermiculite and flushed daily with a complete nutrient solution containing 14.5 mM nitrogen. Water stress was imposed on Essex plants by terminating the application of the nutrient solution. Leaf gas exchange measurements were made on terminal leaflets of third trifoliolate leaves a few days after area expansion was complete.

Leaf gas exchange measurements were made using a LI-6400 portable photosynthesis system (LI-Cor, Inc., Lincoln, NE, USA). Leaf temperature was controlled at 25 °C, and the leaf to air water vapour pressure difference was controlled between 1.2 and 1.4 kPa by manipulating the water vapour pressure of the incoming air stream. Measurements were made on 6 cm^2 sections of intact leaflets at a PPFD of 1500 μmol m^{-2} s^{-1} provided by red and blue light-emitting diodes. Steady-state rates of assimilation (A) in both 2 and 21% $[O_2]$ were recorded at different $[CO_2]$. The 2% $[O_2]$ gas was obtained by blending N_2 with air, using mass flow controllers, and air was assumed to be 21% O_2. The system software was used to correct the output of the infrared analysers for the background $[O_2]$ and to calculate C_i. Gas exchange measurements were conducted inside a controlled environment chamber, in which the water vapour pressure was controlled to match (±0.1 kPa) that inside the cuvette. This was found to eliminate the need to correct for water vapour leakage into or out of the cuvette (Rodeghiero, Niinemets & Cescatti 2007). Corrections for CO_2 leakage were made based on the difference between the chamber and cuvette $[CO_2]$, using the manufacturer's protocol.

Preliminary testing of the method of estimating g'_i was conducted using three Fiskey V plants. Steady-state rates of assimilation (A) in both 2 and 21% $[O_2]$ were measured at 4 $[CO_2]$ from about 250 to 700 μmol mol^{-1}.

In the water stress experiment with Essex, prior to the estimate of g_m, rates of assimilation (A) in 21% $[O_2]$ at a range of $[CO_2]$ were recorded and used to determine whether V_{Cmax} or J was limiting at the $[CO_2]$ used to estimate g_m. The measurement sequence for water-stressed plants deliberately included a large step decrease in external $[CO_2]$, so that it could be determined whether stomatal reopening caused by the switch to low C_i caused a shift in the A versus C_i curve. Estimates of g_m were then obtained by equilibrating leaflets at the desired $[CO_2]$ in 21% $[O_2]$ until gas exchange rates were constant. The $[O_2]$ of the inlet air stream was then switched to 2% while maintaining the same external $[CO_2]$. The rate of photosynthesis at 2% $[O_2]$ and C_i were then recorded when stable, but before stomatal conductance (g_s) responded to the change in $[O_2]$, i.e. within 2 to 3 min. Stomatal conductance increased after a few minutes exposure to 2% $[O_2]$, presumably because C_i was reduced by the increase in A. This increase in stomatal conductance and C_i was used to determine whether A was limited by V_{Cmax} or by J at 2% [O2] by applying the photosynthesis model to the observed increase in A with C_i. After leaf gas exchange measurements were completed on a given leaf, water potential was determined using dew point hygrometry (Wescor HR-33T, Wescor, Inc., Logan, UT, USA) on a disc excised from that leaf.

Values of g_m measured at an external $[CO_2]$ of 380 ± 5 μmol mol^{-1} were determined for unstressed leaves of Essex, and for leaves measured on the third or on the

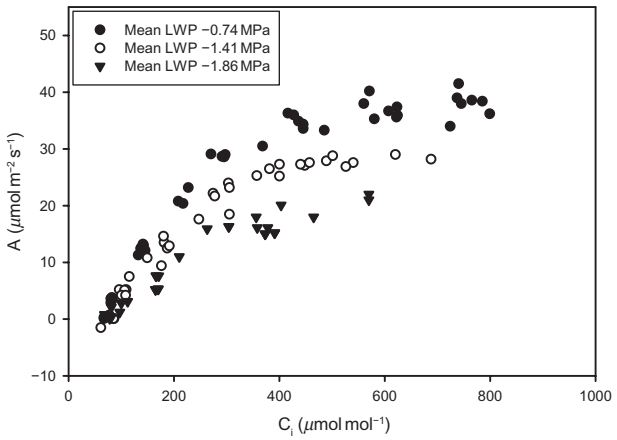

Figure 3. Net CO_2 assimilation rate (A) in relation to substomatal $[CO_2]$ (C_i) for Essex soybean leaves at three levels of stress, defined by leaf water potential (LWP). There were six or seven replicate leaves at each level of stress.

fifth day without nutrient solution application. Measurements were made on leaves of six or seven different plants under each stress condition.

In a subset of three unstressed and three severely stressed leaves, the g_m was also estimated at C_i values of 150 ± 10 and 400 ± 15 μmol mol^{-1} $[CO_2]$. The lower C_i value was chosen to be high enough that estimates of g_m were still insensitive to possible changes in respiration in the light with $[O_2]$ (Tcherkez *et al.* 2008). The upper value of C_i was chosen because, for some leaves, assimilation rates became insensitive to C_i at higher C_i values, which would invalidate the method of estimating g_m from the O_2 response of photosynthesis. A possible dependence of g_m on g_s in unstressed leaves, which happened to vary by a factor of 3 in g_s at 380 μmol mol^{-1} $[CO_2]$, was tested by calculating the correlation between g_m and g_s among leaves.

A Farquhar-type C_3 photosynthesis model with updated kinetic parameters (Sharkey *et al.* 2007) was used to estimate g_m from A and C_i at 2 and 21% $[O_2]$. This was done separately for each leaf by determining, by trial and error, values for V_{Cmax} (or J) and g_m that fit the observed rates of A at both 21 and 2% $[O_2]$ at a given external $[CO_2]$. Values of g_m were resolved to the nearest 0.01 mol m^{-2} s^{-1}. The V_{Cmax} values presented are based on C_c, not on C_i. It was assumed that respiration rate did not change with water stress, based on observations of Ribas-Carbo *et al.* (2005), and we used their value for respiration (0.5 μmol m^{-2} s^{-1}) in the photosynthesis model.

RESULTS

Water stress progressively reduced both the initial slope of the A versus C_i curves and A at high C_i (Fig. 3). For stressed leaves measured at low C_i, data obtained before and after g_s increased at low C_i fit on the same A versus C_i curve. The C_i at 380 μmol mol^{-1} external $[CO_2]$ averaged about 290 μmol mol^{-1} for unstressed leaves [mean leaf water potential (LWP) –0.74 MPa], 200 μmol mol^{-1} in moderately stressed leaves (mean LWP –1.41 MPa) and 180 μmol mol^{-1} in severely stressed leaves (mean LWP –1.86 MPa). At these C_i values, A was always limited by V_{Cmax} at both $[O_2]$. At the moderate level of stress, g_m measured at 380 μmol mol^{-1} $[CO_2]$ was unchanged compared with unstressed leaves, while g_s was reduced by about 80% and V_{Cmax} was reduced by about 25% (Table 2). Under the more severe stress, g_m was reduced by about 50%, with larger reductions in g_s and in V_{Cmax}. The three stress levels also differed significantly in LWP. C_c values averaged about 0.69 to 0.78 of C_i at the different stress levels (Table 2).

The C_i range of 150 to 400 μmol mol^{-1} did not significantly affect g_m either for unstressed or severely stressed leaves, based on paired *t*-tests for measurements made at each $[CO_2]$ level for each leaf (Table 3). For unstressed leaves, g_m varied much less from leaf to leaf than did g_s (see standard errors in Table 2). There was no significant correlation ($r^2 = 0.14$, $n = 7$ leaves) between g_m and g_s in unstressed leaves (not shown).

Table 3. Effect of $[CO_2]$ on internal conductance to CO_2 (g_m) in unstressed and severely stressed leaves of soybean

	g_m (mol m^{-2} s^{-1})	
Stress level	$C_i = 150$ μmol mol^{-1}	$C_i = 400$ μmol mol^{-1}
Unstressed	0.30 ± 0.03a	0.25 ± 0.04a
Severe	0.13 ± 0.04a	0.14 ± 0.03a

At each stress level three leaves were measured at both substomatal $[CO_2]$ (C_i) levels. Values are means (±SE) of three leaves. Values within rows followed by the same letter were not significantly different at $P = 0.05$ by paired *t*-test.

Table 2. Mean (±SE) values of leaf water potential (LWP), stomatal conductance (g_s) to water vapour measured at 380 μmol mol^{-1} $[CO_2]$, maximum rate of carboxylation of Rubisco (V_{Cmax}), internal conductance to CO_2 (g_m) and the $[CO_2]$ at the site of carboxylation (C_c) for soybean leaves at three levels of water stress

LWP (MPa)	g_s (mol m^{-2} s^{-1})	V_{Cmax} (μmol m^{-2} s^{-1})	g_m (mol m^{-2} s^{-1})	C_c (μmol mol^{-1})
–0.74 ± 0.02a	0.690 ± 0.094a	160 ± 10a	0.27 ± 0.01a	200 ± 15a
–1.41 ± 0.05b	0.120 ± 0.014b	120 ± 8b	0.30 ± 0.02a	148 ± 10b
–1.86 ± 0.08c	0.048 ± 0.007c	45 ± 12c	0.12 ± 0.02b	143 ± 13b

Each value is a mean for 6 or 7 leaves from different plants. Within a column, values followed by different letters were significantly different at $P = 0.05$ by analysis of variance.

DISCUSSION

The mean value of g_m at 25 °C estimated from the O_2 sensitivity of photosynthesis in unstressed and moderately stressed Essex soybean leaves was 0.29 mol m^{-2} s^{-1}, which compares with an overall mean value of approximately 0.30 (mean values from 0.20 to 0.40 on different days) at the same temperature estimated from fig. 7 in Bernacchi *et al.* (2005) obtained using fluorescence combined with CO_2 exchange, and 0.32 reported by Gillon & Yakir (2000) using O_2 isotope discrimination. Thus, the O_2 sensitivity method seems to produce reliable estimates of g_m. One limitation of the method is that $[CO_2]$ must remain limiting to net CO_2 fixation, which may not be the case at very low temperatures or at very high $[CO_2]$ (Sage & Kubien 2007). An important procedural note is that when using absolute infrared analysers to measure CO_2 and H_2O exchange rates, as do many commercially available photosynthesis systems, the shift in sensitivity of the analysers because of background $[O_2]$ needs to be accounted for (Bunce 2002), as the LI-6400 software does.

There was no correlation between g_m and g_s in unstressed soybean leaves, as Warren (2008a) also found in three species when manipulating g_s by changing the leaf to air water vapour pressure difference (D). These results indicate that g_m does not directly scale with g_s. As noted by Warren (2008a), the lack of correlation between g_m and g_s also indicates that g_m was insensitive to the changes in C_i resulting from the different g_s. In the case of soybean C_i at 380 μmol mol^{-1}, external $[CO_2]$ varied by about 40 μmol mol^{-1} from leaf to leaf because of the range of g_s, which is similar to the C_i range reported by Warren (2008a). In soybean, there was no significant change in g_m even over a 250 μmol mol^{-1} range of C_i values, both for unstressed leaves and for severely stressed leaves. Loreto *et al.* (1992) and Bunce (2008) also found no change in g_m with C_i, whereas a significant decrease at high C_i has been reported in some species (Centritto *et al.* 2003; During 2003; Flexas *et al.* 2007). However, in many of these cases, changes in g_m over the range of C_i studied here (150 to 400 μmol mol^{-1}) were relatively small, and it is possible that higher C_i values would have resulted in lower g_m in soybean.

It is clear that soil water deficits can substantially reduce g_m in soybean, as also reported in several other species (reviewed in Warren 2008b). In soybean, g_m was much less sensitive than g_s to water stress, with no change in g_m observed at a stress level, which reduced g_s by about 80%. However, further reductions in LWP and g_s were accompanied by a substantial reduction in g_m in soybean. Similar to these results in soybean, Warren (2008a, fig. 5) also found no reduction in g_m with mild soil water stress, which decreased g_s by about 60% in tomato, but a reduction in g_m with more severe stress. In some species, all changes in g_s during drought were accompanied by changes in g_m (Galmes *et al.* 2007; Warren 2008a). Reasons for diverse relationships between g_m and g_s during water stress are unknown, but could be methodological, or related to variation among species in factors contributing to g_m (e.g. anatomical versus various biochemical factors).

In the case of soybean, the relative decrease in g_m was more similar to the decrease in the photosynthetic parameter V_{Cmax} than to the decrease in g_s. Two factors often cast doubt on apparent reductions in V_{Cmax} during water stress, errors in C_i caused by overestimating g_s as it approaches the value of cuticular conductance and errors in C_i caused by patchy stomatal closure during stress. However, in the present case, both these potential errors appear to have been minor, because it was observed that after switching leaves from high to low external $[CO_2]$, the same A versus C_i curve was defined by data before and after g_s more than doubled in response to low C_i. If either patchy closure had occurred or cuticular conductance was significant relative to g_s, then stomatal opening at low C_i would have caused an upward shift in the A versus C_i curve.

Drought is one of the most important environmental factors reducing the yield of crops. It reduces yield partly by reducing the efficiency by which intercepted light is converted into plant material through photosynthesis. The inhibition of photosynthesis during drought is highly correlated with reduced stomatal conductance. There has been a long and still unresolved debate about the existence and the importance of factors other than stomatal closure in limiting photosynthesis during drought. Efforts to improve photosynthesis during drought should be based on knowledge of what physiological processes actually limit photosynthesis.

From the earliest gas exchange measurements of leaves during drought (e.g. Brix 1962), it was evident that progressive drought generally causes approximately parallel reductions in g_s and A. Rather than proving stomatal control of photosynthesis as firstly assumed, however, a truly parallel response would indicate a constant value of substomatal carbon dioxide concentration (C_i). Hence, drought often reduces A at nearly constant C_i, or at least A at any given C_i value. This seemed clear evidence for non-stomatal inhibition of photosynthesis (Farquhar & Sharkey 1982).

This analysis was upset by the realization that the model that calculates C_i from A and g_s may not be valid during drought, because it assumes uniform C_i across the leaf surface. If a substantial fraction of the reduction in stomatal conductance occurs by complete closure of stomata in patches, then an apparently constant C_i can be an artefact of the model (Bunce 1988; Buckley, Farquhar & Mott 1997). Fluorescence measurements indicated a reduction in C_i during drought despite a constant calculated value of C_i (e.g. Downton, Loveys & Grant 1988), thus pointing towards patchy stomatal closure and stomatal control of photosynthesis. However, Osmond *et al.* (1999) found fluorescence signals suggesting low C_c in stressed plants, while observations of guard cells did not indicate patchy closure, thus raising questions about the interpretation of fluorescence signals as indicating patchy closure in stressed plants. Nevertheless, combined fluorescence and gas exchange measurements during drought on a variety of species led to the generalization that mild and moderate water stress reduced photosynthesis only by closing stomata, but severe

stress resulted in non-stomatal inhibition (reviewed in Flexas *et al.* 2004). This interpretation needs to be revisited because low carbon dioxide concentrations at the site of carboxylation during drought could potentially occur without stomatal closure, by a decrease in g_m. A low C_c can neither be taken as evidence of patchy stomatal closure and stomatal limitation of photosynthesis, nor can the ability to overcome the inhibition by very high carbon dioxide levels. In soybean, because g_m was much less reduced by stress than was g_s, C_c values remained 70 to 80% of C_i even under water stress, but other species may differ in this regard and have low C_c during stress. The method of estimating g_m from the oxygen response of photosynthesis may provide estimates of g_m and C_c not subject to the uncertainties of other methods, and allow clearer separation of stomatal and non-stomatal effects of water stress on photosynthesis.

REFERENCES

Bernacchi C.J., Morgan P.B., Ort D.R. & Long S.P. (2005) The growth of soybean under free air [CO_2] enrichment (FACE) stimulates photosynthesis while decreasing *in vivo* Rubisco capacity. *Planta* **220,** 434–446.

Bongi G. & Loreto F. (1989) Gas exchange properties of salt-stressed olive (*Olea europa* L.) leaves. *Plant Physiology* **90,** 1408–1416.

Brix H. (1962) The effect of water stress on the rates of photosynthesis and respiration in tomato and loblolly pine seedlings. *Physiologia Plantarum* **15,** 10–20.

Buckley T.N., Farquhar G.D. & Mott K.A. (1997) Qualitative effects of patchy stomatal conductance distribution features on gas-exchange calculations. *Plant, Cell & Environment* **20,** 867–880.

Bunce J.A. (1988) Nonstomatal inhibition of photosynthesis by water stress. Reduction in photosynthesis at high transpiration rate without stomatal closure in field-grown tomato. *Photosynthesis Research* **18,** 357–362.

Bunce J.A. (2002) Sensitivity of infrared water vapor analyzers to oxygen concentration and errors in stomatal conductance. *Photosynthesis Research* **71,** 273–276.

Bunce J.A. (2008) Acclimation of photosynthesis to temperature in *Arabidopsis thaliana* and *Brassica oleracea*. *Photosynthetica* **46,** 517–524.

Centritto M., Loreto F. & Chartzoulakis K. (2003) The use of low [CO_2] to estimate diffusional and non-diffusional limitation of photosynthetic capacity of salt-stressed olive saplings. *Plant, Cell & Environment* **26,** 585–594.

Di Marco G., Manes F., Tricoli D. & Vitale E. (1990) Fluorescence parameters measured concurrently with net photosynthesis to investigate chloroplastic CO_2 concentration in leaves of *Quercus ilex* L. *Journal of Plant Physiology* **136,** 538–543.

Downton W.J.S., Loveys B.R. & Grant W.J.R. (1988) Non-uniform stomatal closure induced by water stress causes putative non-stomatal inhibition of photosynthesis. *New Phytologist* **110,** 503–509.

During H. (2003) Stomatal and mesophyll conductances control CO_2 transfer to chloroplasts in leaves of grapevine (*Vitis vinifera* L.). *Vitis* **42,** 65–68.

Ethier G.J. & Livingston N.J. (2004) On the need to incorporate sensitivity to CO_2 transfer conductance into the Farquhar-von Caemmerer-Berry leaf photosynthesis model. *Plant, Cell & Environment* **27,** 137–153.

Ethier G.J., Livingston N.J., Harrison D.L., Black T.A. & Moran J.A. (2006) Low stomatal and internal conductance to CO_2 versus Rubisco deactivation as determinants of the photosynthetic decline of ageing evergreen leaves. *Plant, Cell & Environment* **29,** 2168–2184.

Evans J.R., Sharkey T.D., Berry J.A. & Farquhar G.D. (1986) Carbon isotope discrimination measured concurrently with gas exchange to investigate CO_2 diffusion in leaves of higher plants. *Australian Journal of Plant Physiology* **13,** 281–292.

Farquhar G.D. & Sharkey T.D. (1982) Stomatal conductance and photosynthesis. *Annual Review of Plant Physiology* **33,** 317–345.

Farquhar G.D., von Caemmerer S. & Berry J.A. (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149,** 78–90.

Flexas J., Bota J., Loreto F., Cornic G. & Sharkey T.D. (2004) Diffusive and metabolic limitations to photosynthesis under drought and salinity in C_3 plants. *Plant Biology* **6,** 269–279.

Flexas J., Diaz-Espejo A., Galmes J., Kaldenhof R., Medrano H. & Ribas-Carbo M. (2007) Rapid variations of mesophyll conductance in response to changes in CO_2 concentration around leaves. *Plant, Cell & Environment* **30,** 1284–1298.

Flexas J., Ribas-Carbo M., Diaz-Espejo A., Galmes J. & Medrano H. (2008) Mesophyll conductance to CO_2: current knowledge and future prospects. *Plant, Cell & Environment* **31,** 602–621.

Galmes J., Medrano H. & Flexas J. (2007) Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. *New Phytologist* **175,** 81–93.

Gillon J.S. & Yakir D. (2000) Internal conductance to CO_2 diffusion (COO)-O-18 discrimination in C-3 leaves. *Plant Physiology* **123,** 201–213.

Harley P.C., Loreto F., Di Marco G. & Sharkey T.D. (1992) Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by the analysis of the response of photosynthesis to CO_2. *Plant Physiology* **98,** 1429–1436.

Lichtenthaler H.K., Buschmann C. & Knapp M. (2005) How to correctly determine the different chlorophyll fluorescence parameters and the chlorophyll fluorescence decrease ratio R_{Fd} of leaves with the PAM fluorometer. *Photosynthetica* **43,** 379–393.

Loreto F., Harley P.C., Di Marco G. & Sharkey T.D. (1992) Estimation of mesophyll conductance to CO_2 flux by three different methods. *Plant Physiology* **98,** 1437–1443.

Osmond C.B., Kramer D. & Luttge U. (1999) Reversible, water-stress induced non-uniform chlorophyll fluorescence quenching in wilting leaves of *Potentilla reptans* may not be due to patchy stomatal responses. *Plant Biology* **1,** 618–624.

Ribas-Carbo M., Taylor N.L., Giles L., Busquets S., Finnegan P.M., Day D.A., Lambers H., Medrano H., Berry J.A. & Flexas J. (2005) Effects of water stress on respiration in soybean leaves. *Plant Physiology* **139,** 466–473.

Rodeghiero M., Niinemets U. & Cescatti A. (2007) Major diffusion leaks of clamp-on cuvettes still unaccounted: how erroneous are the estimates of Farquhar *et al.* model parameters? *Plant, Cell & Environment* **30,** 1006–1022.

Sage R.F. & Kubien D.S. (2007) The temperature response of C_3 and C_4 photosynthesis. *Plant, Cell & Environment* **30,** 1086–1106.

Sharkey T.D., Bernacchi C.J., Farquhar G.D. & Singsaas E.L. (2007) Fitting photosynthetic carbon dioxide response curves for C_3 leaves. *Plant, Cell & Environment* **30,** 1035–1040.

Singsass E.L., Ort D.R. & DeLucia E.H. (2003) Elevated CO_2 effects on mesophyll conductance and its consequences for interpreting photosynthetic physiology. *Plant, Cell & Environment* **27,** 41–50.

Tcherkez G., Bligny R., Gout E., Mahe A., Hodges M. & Cornic G. (2008) Respiratory metabolism of illuminated leaves depends on CO_2 and O_2 conditions. *Proceedings of the National Academy of Sciences of the United States of America* **105,** 797–802.

Warren C.R. (2006) Estimating the internal conductance to CO_2 movement. *Functional Plant Biology* **33,** 432–442.

Warren C.R. (2008a) Soil water deficits decrease the internal conductance to CO_2 transfer but atmospheric water deficits do not. *Journal of Experimental Botany* **59,** 327–334.

Warren C.R. (2008b) Stand aside stomata, another actor deserves center stage: the forgotten role of the internal conductance to CO_2 transfer. *Journal of Experimental Botany* **59,** 1475–1487.

Warren C.R. & Adams M.A. (2006) Internal conductance does not scale with photosynthetic capacity: implications for carbon isotope discrimination and the economics of water and nitrogen use in photosynthesis. *Plant, Cell & Environment* **29,** 192–201.

Yamori W., Noguchi K., Hanba Y.T. & Terashima I. (2006) Effects in internal conductance on the temperature dependence of the photosynthetic rate in spinach leaves from contrasting growth temperatures. *Plant and Cell Physiology* **47,** 1069–1080.

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